The pGinger family of expression plasmids

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Abstract

The pGinger suite of expression plasmids comprises 43 plasmids that will enable precise constitutive and inducible gene expression in a wide range of gram-negative bacterial species. Constitutive vectors are composed of 16 synthetic constitutive promoters upstream of RFP, with a broad host range BBR1 origin and a kanamycin resistance marker. The family also has seven inducible promoters (Jungle Express, NahR, XylS, RhaS, Lac, LacUV5, and TetR) controlling RFP expression on BBR1/kanamycin plasmid backbones. For four of these inducible promoters (Jungle Express, NahR, Lac, and TetR), we created variants that utilize the RK2 origin and spectinomycin or gentamicin selection. Relevant RFP expression and growth data have been

35 collected in the model bacterium *Escherichia coli* as well as *Pseudomonas putida*. All pGinger
36 vectors are available via the Joint BioEnergy Institute (JBEI) Public Registry.

37 **Introduction**

38 Precise and reliable control over gene expression is one of the most fundamental
39 requirements of synthetic biology (1). Consequently, there has been considerable effort towards
40 identifying myriad genetic elements that enable researchers to regulate the strength and timing of
41 transcription across all domains of life (2–4). The end result of these efforts are often
42 consolidated families of plasmid vectors that facilitate advanced genetic engineering, such as the
43 BglBrick family of plasmids for *E. coli* (5, 6) and the jStack vectors used in multiple plant
44 species (7). However, as the field of synthetic biology moves beyond traditional model
45 organisms, families of expression vectors must be tailored to meet the specific requirements of
46 particular hosts. Advances in non-model organisms often come in the form of species or genus
47 specific toolkits (8, 9), though more recently comprehensive plasmid toolkits have been
48 developed and validated for a wide range of gram-negative organisms (10). Still, given that many
49 bacteria require very particular combinations of promoters, origins, and selectable markers to
50 enable controlled gene expression, there remains a need for vectors that will allow rapid
51 prototyping of genetic circuits in understudied bacteria.

52 To facilitate the exploration of non-model hosts, we have developed a small suite of
53 plasmids that permit both constitutive and inducible expression from the broad host-range origin
54 of replication BBR1 using a kanamycin selection marker. For a subset of the inducible promoters
55 that are known to work across multiple hosts, we have assembled combinatorial variants that
56 utilize the compatible broad host-range origin RK2 (11) as well as both spectinomycin and
57 gentamicin selection markers. This family of plasmids, which we have named the pGinger suite,

requires no assembly of these parts, can be easily cloned into via standard Gibson assembly techniques, and has both digital sequences and physical samples that can be publicly accessed through the Joint BioEnergy Institute (JBEI) registry (12).

Results

Design and Architecture of pGinger Plasmids

All pGinger vectors express RFP with a consensus ribosomal binding site (RBS - TTTAAGAAGGAGATATACAT) derived from the BglBrick plasmid library. The overall conserved plasmid architecture and naming convention of the pGinger suite are shown in **Figure 1**.

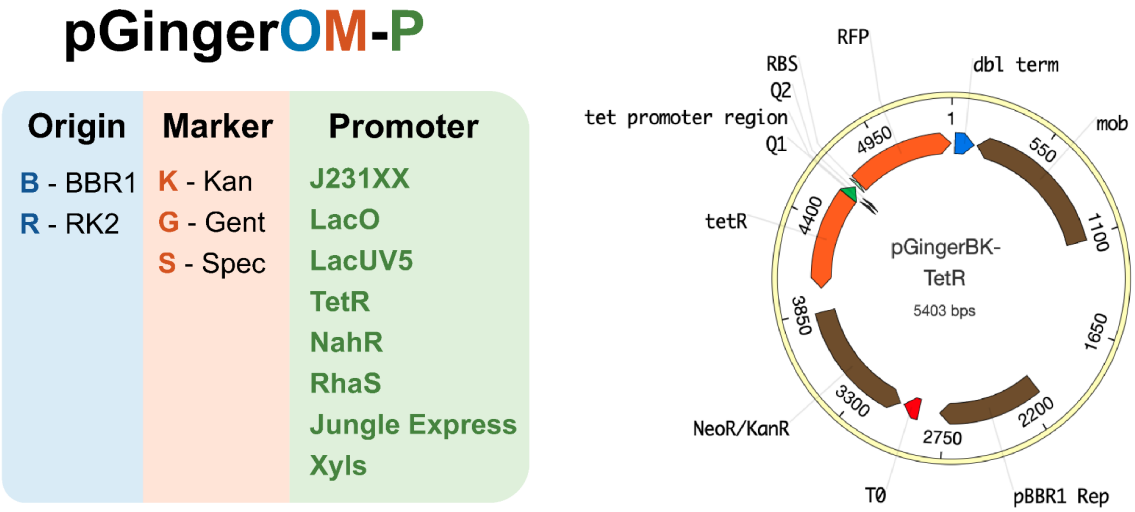

Figure 1: **Plasmids architecture of the pGinger suite**: The pGinger plasmids share a common naming convention where the first two letters after pGinger correspond to the origin and resistance marker respectively, followed by the promoter. All plasmids share the same architecture as the above map of pGingerBK-TetR, whereby a conserved RBS-RFP is downstream of the promoter followed by a strong terminator. All selectable markers are upstream of the promoter, with the origin between the marker and the RFP cassette.

74 The BBR1 origin and kanamycin cassette of relevant pGinger vectors were both derived from
75 plasmid pBADTrfp (13). To develop a family of constitutive expression plasmids, the AraC
76 coding sequence and promoter of pBADTrfp were replaced with 16 different synthetic promoters
77 from the Anderson Promoter Library (http://parts.igem.org/Promoters/Catalog/Anderson). For
78 the inducible vectors, the AraC coding sequence and promoter of pBADTrfp were replaced with
79 the following seven inducible systems: Jungle Express - derived from pTR_sJExD-rfp (14);
80 NahR - derived from pPS43 (15); RhaS - derived from pCV203 (15); TetR - derived from
81 pBbE2a-RFP; XylS - derived from pPS66 (15); Lac - derived from pBbE6a-RFP (6); LacUV5 -
82 derived from pBbE5a-RFP (6). For four of the inducible promoters (Jungle Express, NahR, Lac,
83 and TetR), additional vectors were constructed that varied both the origin and antibiotic marker.
84 All RK2 origins were derived from pBb(RK2)1k-GFPuv (8), while the gentamicin resistance
85 cassette was derived from pMQ30 (16), and the spectinomycin cassette was derived from
86 pSR43.6 (17). All BBR1 derivatives of pBADTrfp contain the *mob* element that facilitates
87 conjugal transfer. A full description of each pGinger vector can be found in **Table 1**.
88
89

Name	Origin	Marker	Promoter Class	Promoter	Inducer	JBEI ICE No.
pGingerBK-J23100	BBR1	Kanamycin	Constitutive	J23100	NA	JPUB_020797
pGingerBK-J23101	BBR1	Kanamycin	Constitutive	J23101	NA	JPUB_020799
pGingerBK-J23102	BBR1	Kanamycin	Constitutive	J23102	NA	JPUB_020815
pGingerBK-J23103	BBR1	Kanamycin	Constitutive	J23103	NA	JPUB_020801
pGingerBK-J23104	BBR1	Kanamycin	Constitutive	J23104	NA	JPUB_020803
pGingerBK-J23105	BBR1	Kanamycin	Constitutive	J23105	NA	JPUB_020817
pGingerBK-J23106	BBR1	Kanamycin	Constitutive	J23106	NA	JPUB_020793
pGingerBK-J23107	BBR1	Kanamycin	Constitutive	J23107	NA	JPUB_020819
pGingerBK-J23108	BBR1	Kanamycin	Constitutive	J23108	NA	JPUB_020821

pGingerBK-J23110	BBR1	Kanamycin	Constitutive	J23110	NA	JPUB_020805
pGingerBK-J23111	BBR1	Kanamycin	Constitutive	J23111	NA	JPUB_020807
pGingerBK-J23113	BBR1	Kanamycin	Constitutive	J23113	NA	JPUB_020809
pGingerBK-J23114	BBR1	Kanamycin	Constitutive	J13114	NA	JPUB_020811
pGingerBK-J23117	BBR1	Kanamycin	Constitutive	J13117	NA	JPUB_020795
pGingerBK-J23118	BBR1	Kanamycin	Constitutive	J23118	NA	JPUB_020823
pGingerBK-J23119	BBR1	Kanamycin	Constitutive	J23119	NA	JPUB_020813
pGingerBK-JE	BBR1	Kanamycin	Inducible	Jungle Express	Crystal Violet	JPUB_020825
pGingerBK-NahR	BBR1	Kanamycin	Inducible	NahR	Salicylic acid	JPUB_020831
pGingerBK-RhaS	BBR1	Kanamycin	Inducible	RhaS	Rhamnose	JPUB_020829
pGingerBK-TetR	BBR1	Kanamycin	Inducible	TetR	Oxytetracycline	JPUB_020835
pGingerBK-XylS	BBR1	Kanamycin	Inducible	XylS	Benzoate	JPUB_020827
pGingerBK-Lac	BBR1	Kanamycin	Inducible	Lac	IPTG	JPUB_020833
pGingerBK-LacUV5	BBR1	Kanamycin	Inducible	LacUV5	IPTG	JPUB_020837
pGingerBG-JE	BBR1	Gentamicin	Inducible	Jungle Express	Crystal Violet	JPUB_020847
pGingerBS-JE	BBR1	Spectinomycin	Inducible	Jungle Express	Crystal Violet	JPUB_020855
pGingerRK-JE	RK2	Kanamycin	Inducible	Jungle Express	Crystal Violet	JPUB_020871
pGingerRG-JE	RK2	Gentamicin	Inducible	Jungle Express	Crystal Violet	JPUB_020881
pGingerRS-JE	RK2	Spectinomycin	Inducible	Jungle Express	Crystal Violet	JPUB_020863
pGingerBG-NahR	BBR1	Gentamicin	Inducible	NahR	Salicylic acid	JPUB_020845
pGingerBS-NahR	BBR1	Spectinomycin	Inducible	NahR	Salicylic acid	JPUB_020853
pGingerRK-NahR	RK2	Kanamycin	Inducible	NahR	Salicylic acid	JPUB_020869
pGingerRG-NahR	RK2	Gentamicin	Inducible	NahR	Salicylic acid	JPUB_020879
pGingerRS-NahR	RK2	Spectinomycin	Inducible	NahR	Salicylic acid	JPUB_020859
pGingerBG-TetR	BBR1	Gentamicin	Inducible	TetR	Oxytetracycline	JPUB_020843
pGingerBS-TetR	BBR1	Spectinomycin	Inducible	TetR	Oxytetracycline	JPUB_020851
pGingerRK-TetR	RK2	Kanamycin	Inducible	TetR	Oxytetracycline	JPUB_020865
pGingerRG-TetR	RK2	Gentamicin	Inducible	TetR	Oxytetracycline	JPUB_020877
pGingerRS-TetR	RK2	Spectinomycin	Inducible	TetR	Oxytetracycline	JPUB_020861
pGingerBG-Lac	BBR1	Gentamicin	Inducible	Lac	IPTG	JPUB_020841
pGingerBS-Lac	BBR1	Spectinomycin	Inducible	Lac	IPTG	JPUB_020849

pGingerRK-Lac	RK2	Kanamycin	Inducible	Lac	IPTG	JPUB_020867
pGingerRG-Lac	RK2	Gentamicin	Inducible	Lac	IPTG	JPUB_020875
pGingerRS-Lac	RK2	Spectinomycin	Inducible	Lac	IPTG	JPUB_020857

Table 1: **Plasmids in the pGinger suite**: Relevant characteristics of pGinger plasmids including origin of replication, antibiotic selection, promoter characteristics, and if applicable inducing molecule. JBEI public registry numbers are also included for digital accessibility.

Evaluation of Constitutive Expression pGinger Plasmids

To evaluate the relative strength of constitutive Anderson promoters in the context of the pGinger vectors, plasmids were introduced into both *P. putida* and *E. coli*. Fluorescence was measured after growth in LB medium after 24 hours. When fluorescence was normalized to cell density, expression from Anderson promoters showed significant correlation (Spearman's $\rho = 0.49$, $p = 0.045$) between *P. putida* and *E. coli* (**Figure 2**). Promoters J23103 and J23113 were significantly stronger in *E. coli* than in *P. putida*, while promoter J23111 was significantly stronger in *P. putida*. Promoter sequences and mean expression values in both *E. coli* and *P. putida* are listed in **Table 2**.

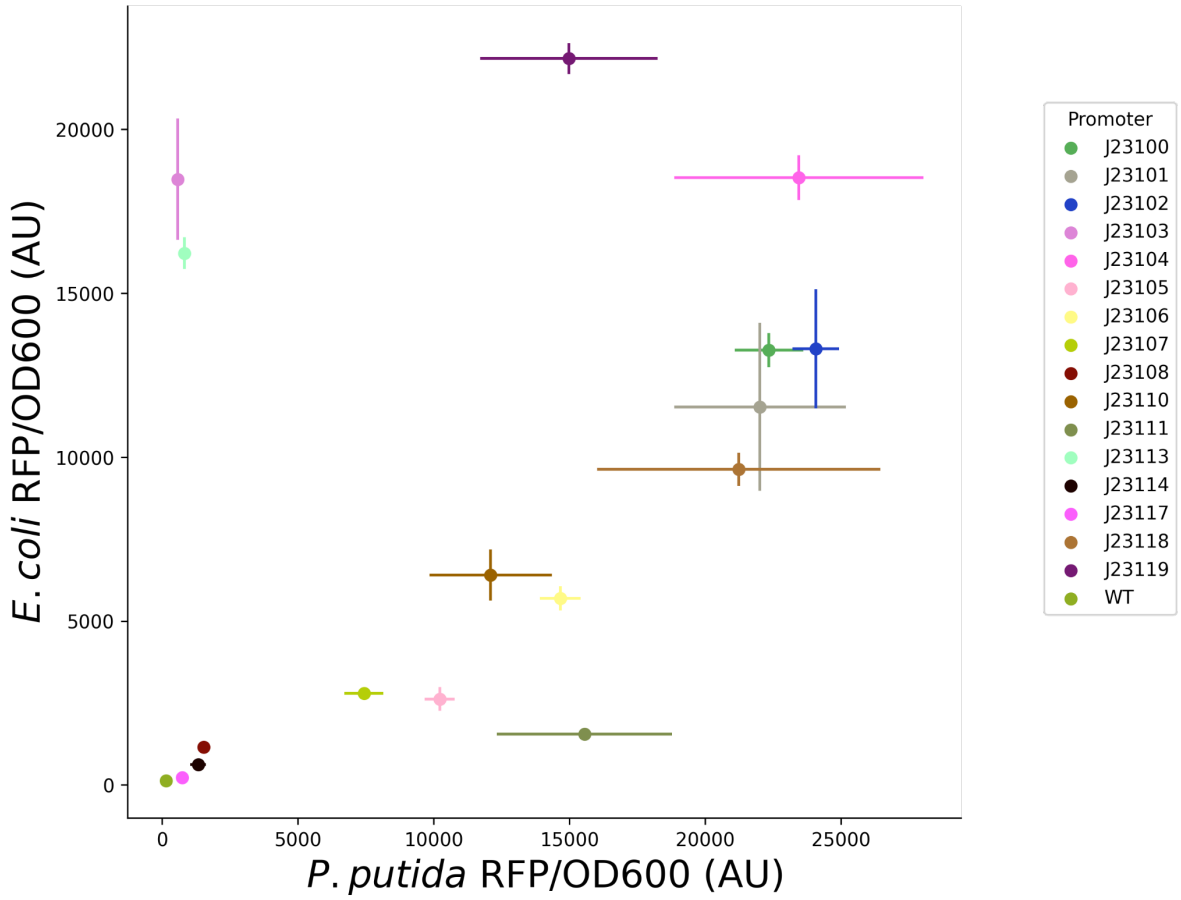

Figure 2: Activity of Constitutive Promoters in *E. coli* and *P. putida*. RFP expression normalized to cell density from Anderson promoters within either *E. coli* (y-axis) or *P. putida* (x-axis) are shown with standard deviations (n=3).

Promoter	Promoter Sequence	*E. coli* expression	*P. putida* expression
J23100	ttgacggctagctcagtcctaggtacagtgctagc	13267 (+/- 517)	22343 (+/- 1262)
J23101	tttacagctagctcagtcctaggtattatgctagc	11530 (+/- 2565)	22010 (+/- 3162)
J23102	ttgacagctagctcagtcctaggtactgtgctagc	13300 (+/- 1815)	24067 (+/- 858)
J23103	ctgatagctagctcagtcctagggattatgctagc	18476 (+/- 1857)	565 (+/- 135)
J23104	ttgacagctagctcagtcctaggtattgtgctagc	18522 (+/- 682)	23440 (+/- 4588)
J23105	tttacggctagctcagtcctaggtactatgctagc	2622 (+/- 363)	10220 (+/- 558)

J23106	tttacggctagctcagtcctaggtatagtgctagc	5697 (+/- 369)	14659 (+/- 748)
J23107	tttacggctagctcagccctaggtattatgctagc	2798 (+/- 44)	7429 (+/- 716)
J23108	ctgacagctagctcagtcctaggtataatgctagc	1149 (+/- 84)	1523 (+/- 84)
J23110	tttacggctagctcagtcctaggtacaatgctagc	6402 (+/- 782)	12098 (+/- 2251)
J23111	ttgacggctagctcagtcctaggtatagtgctagc	1547 (+/- 106)	15548 (+/- 3229)
J23113	ctgatggctagctcagtcctagggattatgctagc	16220 (+/- 480)	826 (+/- 92)
J23114	tttatggctagctcagtcctaggtacaatgctagc	625 (+/- 48)	1325 (+/- 289)
J23117	ttgacagctagctcagtcctagggattgtgctagc	229 (+/- 25)	730 (+/- 28)
J23118	ttgacggctagctcagtcctaggtattgtgctagc	9628 (+/- 507)	21237 (+/- 5215)
J23119	ttgacagctagctcagtcctaggtataatgctagc	22157 (+/- 473)	14979 (+/- 3262)
WT	NA	124 (+/- 6)	141 (+/- 17)

Table 2: Expression of pGinger Anderson promoters: For each Anderson promoter the sequence is provided as well as the mean cell density normalized RFP fluorescence in both *E. coli* and *P. putida*. Standard deviations are provided in parentheses, n=3.

Evaluation of Inducible pGinger Plasmids

The expression of the seven inducible promoters within the pGinger suite was evaluated using the BBR1 origin and kanamycin marker (pGingerBK) against a titration of the inducer in both *E. coli* and *P. putida* (**Figure 3**). All promoters showed inducibility in *E. coli*, and all but the rhamnose inducible RhaS promoter showed inducibility in *P. putida*. Relevant expression characteristics of the inducible pGingerBK vectors in both tested bacteria are listed in **Table 3**. The strongest normalized expression from an inducible promoter in *E. coli* was the TetR system, while both the strongest promoters in *P. putida* were found to be NahR and Jungle Express

promoters, which showed nearly identical maximal expression. In both bacteria, the Jungle Express promoter demonstrated the greatest level of induction relative to background expression.

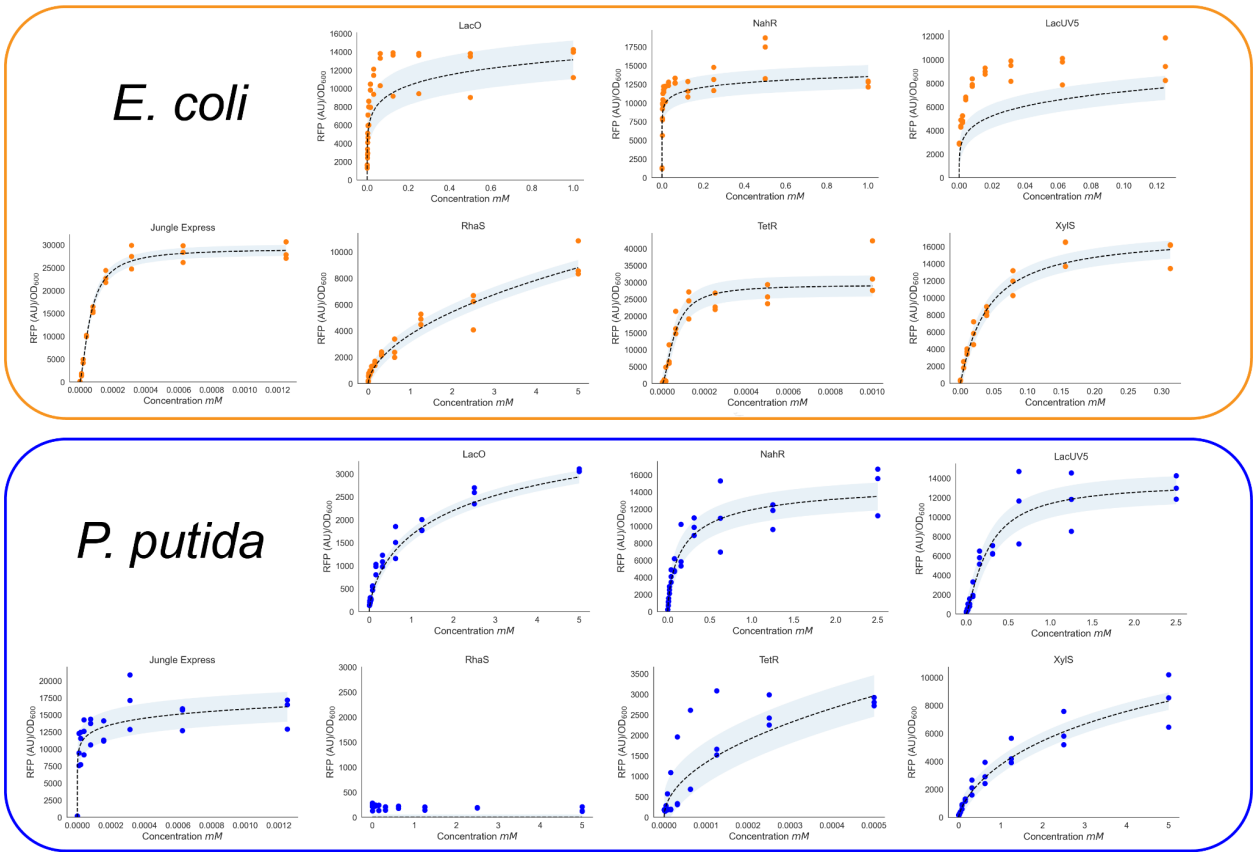

Figure 3: Activity of Inducible Promoters in *E. coli* and *P. putida*. RFP expression normalized to cell density (y-axis) from inducible promoters within either *E. coli* (top panel in orange) or *P. putida* (bottom panel in blue) as a function of inducer concentration in mM (x-axis). Fits to the Hill equation are shown as dashed lines and shaded to show confidence intervals. Raw data points are overlaid (n=3).

Promoter	Organism	Background	Max Exp.	Max Conc.	Induction
LacO	*E. coli*	1510 (+/- 186)	13127 (+/- 1693)	1 mM	9x
	P. putida	137 (+/- 5)	3074 (+/- 30)	5 mM	22x
NahR	*E. coli*	1260 (+/- 57)	16484 (+/- 1693)	500 uM	9x
	P. putida	847 (+/- 244)	25697 (+/- 2976)	5 mM	30x

LacUV5	*E. coli*	2889 (+/- 36)	10137 (+/- 855)	250 uM	4x
	P. putida	203 (+/- 53)	16622 (+/- 3671)	10 mM	82x
Jungle Express	*E. coli*	100 (+/- 2)	28545 (+/- 1890)	125 nM	285x
	P. putida	183 (+/- 1)	16956 (+/- 4002)	313 nM	93x
RhaS	*E. coli*	172 (+/- 42)	9251 (+/- 1389)	5 mM	54x
	P. putida	NA	NA	NA	NA
TetR	*E. coli*	341 (+/- 10)	33631 (+/- 7692)	1 uM	98x
	P. putida	176 (+/- 9)	3214 (+/- 319)	1 uM	18x
XylS	*E. coli*	329 (+/- 57)	15280 (+/- 1590)	313 uM	46x
	P. putida	161 (+/- 7)	8401 (+/- 1877)	5 mM	52x

Table 3: Inducible Promoters in *E. coli* and *P. putida*: For each inducible promoter on a BBR1 origin with a kanamycin marker, the experimentally observed background (uninduced) fluorescence and maximal fluorescence are given for both *E. coli* and *P. putida*. Standard deviations are provided in parentheses, n=3. Additionally, the inducer concentration used to achieve maximal expression and the relative induction levels are listed.

To evaluate the effect of varying origin and selectable markers on expression from inducible promoters, all six variants of the Jungle Express, LacO, NahR, and TetR were investigated for their dose-response to their inducer molecules in *E. coli* (**Figure 4**). Relevant expression parameters are listed in **Table 4**. In general, BBR1 variants showed greater expression than RK2 origin plasmids, which is expected given the higher copy of BBR1 plasmids in *E. coli* (10). Amongst the pGinger Jungle Express vectors, both pGingerRS-JE and pGingerRK-JE showed dose-responses distinct from the other vectors (**Figure 4A**). Notably, all

pGingerRS (RK2-Spectinomycin) plasmids showed the lowest expression across each promoter tested (**Table 4**).

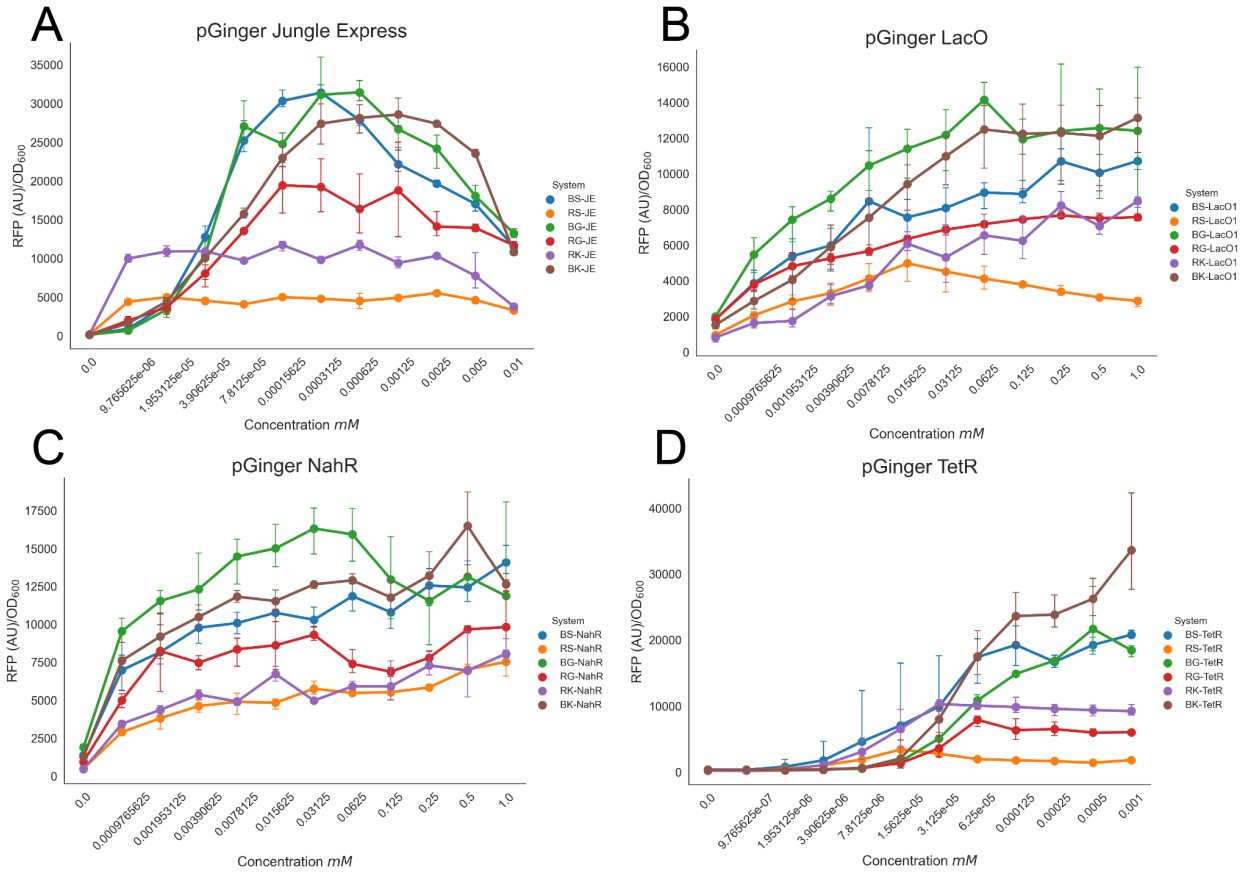

Figure 4: Activity of Inducible pGinger variants in *E. coli*. For origin and selection marker pGinger variants of Jungle Express (A), LacO (B), NahR (C), and TetR (D), dose-response curves of normalized RFP expression are shown as a function of mM inducer. Error bars represent standard deviations (n=3). Note that the x-axis is non-linear.

Promoter	Origin	Marker	Background	Max Exp.	Max Conc.	Induction
LacO	BBR1	Kan	1510 (+/- 186)	13127 (+/- 1693)	1 mM	9x
LacO	BBR1	Gent	1976 (+/- 70)	14143 (+/- 1608)	63 uM	7x
LacO	BBR1	Spec	1830 (+/- 320)	10694 (+/- 943)	250 uM	6x

LacO	RK2	Kan	803 (+/- 216)	8213 (+/- 721)	250 uM	10x
LacO	RK2	Gent	1823 (+/- 78)	7654 (+/- 230)	250 uM	4x
LacO	RK2	Spec	950 (+/- 46)	4967 (+/- 895)	16 uM	5x
NahR	BBR1	Kan	1260 (+/- 57)	16484 (+/- 1693)	500 uM	9x
NahR	BBR1	Gent	1877 (+/- 334)	16317 (+/- 1534)	31 uM	9x
NahR	BBR1	Spec	1372 (+/- 84)	14083 (+/- 984)	1 mM	10x
NahR	RK2	Kan	445 (+/- 39)	8048 (+/- 859)	1 mM	18x
NahR	RK2	Gent	934 (+/- 56)	9299 (+/- 460)	1 mM	10x
NahR	RK2	Spec	482 (+/- 69)	7510 (+/- 817)	1 mM	16x
Jungle Express	BBR1	Kan	100 (+/- 2)	28545 (+/- 1890)	125 nM	285x
Jungle Express	BBR1	Gent	124 (+/- 24)	31409 (+/- 1348)	625 nM	254x
Jungle Express	BBR1	Spec	107 (+/- 9)	31360 (+/- 905)	313 nM	292x
Jungle Express	RK2	Kan	151 (+/- 25)	11702 (+/- 3180)	625 nM	78x
Jungle Express	RK2	Gent	91 (+/- 6)	19413 (+/- 3180)	156 nM	214x
Jungle Express	RK2	Spec	153 (+/- 18)	4978 (+/- 305)	20 nM	32x
TetR	BBR1	Kan	341 (+/- 10)	33631 (+/- 7692)	1 uM	98x
TetR	BBR1	Gent	351 (+/- 32)	21646 (+/- 5579)	500 nM	62x
TetR	BBR1	Spec	232 (+/- 43)	19224 (+/- 3027)	125 nM	83x
TetR	RK2	Kan	184 (+/- 5)	10281 (+/- 967)	31 nM	56x
TetR	RK2	Gent	232 (+/- 39)	7883 (+/- 865)	63 nM	34x
TetR	RK2	Spec	197 (+/- 5)	3399 (+/- 143)	16 nM	17x

150

151 **Table 4: Inducible pGinger variants in *E. coli***: For pGinger variants of LacO, NahR, Jungle Express, and TetR

152 promoters, the experimentally observed background (uninduced) fluorescence and maximal fluorescence in *E. coli*

153 are provided. Standard deviations are provided in parentheses, n=3. Additionally, the inducer concentration used to
154 achieve maximal expression and the relative induction levels are listed.
155
156

157 **Discussion**

158 The pGinger suite of plasmids offers researchers an array of small, pre-assembled vectors
159 that will permit rapid identification of useful genetic elements in diverse gram-negative bacteria
160 due to the use of broad host-range origins (RK2) and selectable markers known to work across
161 many species (kanamycin, spectinomycin, gentamicin). The compatibility of RK2 and BBR1
162 origins may also permit researchers to introduce multiple pGinger vectors into a single strain
163 simultaneously (11). In combination with other recent plasmid suites that have been publicly
164 released, the pGinger plasmids have the potential to facilitate more advanced synthetic biology
165 and metabolic engineering efforts in bacterial species that have been traditionally understudied.

166 **Materials & Methods**

167 Strains and Media

168 Cultures were grown in lysogeny broth (LB) Miller medium (BD Biosciences, USA) at
169 37 °C for *E. coli* XL1-Blue (QB3 Macrolab, USA) and 30 °C for *P. putida* KT2440 (ATCC
170 47054). The medium was supplemented with kanamycin (50 mg/L, Sigma Aldrich, USA),
171 gentamicin (30 mg/L, Fisher Scientific, USA), or spectinomycin (100mg/L, Sigma Aldrich,
172 USA), when indicated. All other compounds were purchased through Sigma Aldrich (Sigma
173 Aldrich, USA).

174 Plasmid Design and Construction

175 All plasmids were designed using Device Editor and Vector Editor software, while all
176 primers used for the construction of plasmids were designed using j5 software (12, 18, 19).
177 Plasmids were assembled via Gibson Assembly using standard protocols (20). Plasmids were
178 routinely isolated using the Qiaprep Spin Miniprep kit (Qiagen, USA), and all primers were
179 purchased from Integrated DNA Technologies (IDT, Coralville, IA).

180 Plasmid and Sequence Availability

181 All strains and plasmids from Table 1 can be found via the following link to the JBEI
182 Public Registry: https://public-registry.jbei.org/folders/771.

183 Characterization assays

184 To characterize RFP expression from these vectors, we measured optical density and
185 fluorescence after growth in 96 well plates for 24 hours. First, overnight cultures were inoculated
186 into 5 mL of LB medium from single colonies and grown at 30 °C or 37 °C. These cultures were
187 then diluted 1:100 into 500 µL of LB medium with the appropriate antibiotic in 96 square v-
188 bottom deep well plates (Biotix™ DP22009CVS). For characterization of the inducible systems,
189 inducer was added to wells in the first column of the plate at the maximum concentration tested
190 and diluted two-fold across the plate until the last column, which was left as the zero-inducer
191 control. Plates were sealed with a gas-permeable microplate adhesive film (Axygen™ BF400S)
192 and grown for 24 hours at either 30 °C or 37 °C with shaking at 200 rpm. Optical density was
193 measured at 600 nm, and fluorescence was measured at an excitation wavelength of 535 nm and
194 an emission wavelength of 620 nm. All data was analyzed and visualized using custom Python
195 scripts using the SciPy (22), NumPy (23), Pandas, Matplotlib, and Seaborn libraries. Fits to the
196 Hill equation were done as previously described (24).

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217 **Contributions**

218 Conceptualization; A.N.P., M.G.T.; Methodology; A.N.P., M.G.T..; Investigation, A.N.P.,
219 M.G.T., L.D.K., K.M.V., L.M.W.; Writing – Original Draft, A.N.P., M.G.T..; Writing – Review
220 and Editing, All authors.; Resources and supervision; P.M.S, J.D.K.

221 **Competing Interests**

222 J.D.K. has financial interests in Amyris, Ansa Biotechnologies, Apertor Pharma, Berkeley Yeast,
223 Demetrix, Lygos, Napigen, ResVita Bio, and Zero Acre Farms.

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